

RESEARCH REPORT

Transcriptional effect of serotonin in the ganglia of *Lymnaea stagnalis***C Benatti^{1,2}, C Colliva¹, JMC Blom^{2,3}, E Ottaviani¹, F Tascetta^{1,2}**¹ Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy² Center for Neuroscience and Neurotechnology University of Modena and Reggio Emilia, Modena, Italy³ Department of Education and Humanities University of Modena and Reggio Emilia, Modena, Italy

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Abstract

The serotonin system (5HT) is highly conserved in both vertebrates and invertebrates, and numerous evidence supports a biological link between 5HT and numerous animal function. In the present paper we evaluated the transcriptional effects of a serotonergic stimulation on selected targets involved in 5HT signalling and neurotransmission in the central nervous system of the great pond snail *Lymnaea stagnalis*. Adult snails were treated acutely (6 h) or chronically (48 h) with either 5-hydroxytryptophan (5-HTP 1mM), the immediate precursor of serotonin, fluoxetine (FLX 1μM), a selective serotonin reuptake inhibitor, or a combination of two. The central ring ganglia were dissected and used for q-PCR gene expression analysis. Transcription was strongly induced following a chronic, but not an acute, exposure to 5-HTP in the ganglia of *Lymnaea*. In particular, *LymCREB1* and *LymP2X* mRNA levels were decreased following a 6 h exposure and increased in snails receiving 5-hydroxytryptophan for 48 h. Interestingly, this effect was reduced when snails were exposed chronically to both 5-HTP and FLX, suggesting a role for SERT in mediating the effect of 5-hydroxytryptophan. These data suggest that *L. stagnalis* is suited to unravel the complexity of the serotonin signaling pathway.

Key Words: serotonin; CREB; *Lymnaea stagnalis***Introduction**

The serotonin is an ancestral complex neurotransmitter system that plays an important role in the regulation of many biological functions. Serotonin has a fundamental role in the modulation of stress-induced excitability (arousal), in the defensive behavior (Il-Han *et al.*, 2010), in the modulation of aggressive behaviors and in the control of anxiety.

Normally, for these studies, have been used small mammals (*i.e.*, rats and mice) but this approach may not be always effective and is accompanied by many ethical and economical drawbacks (Tascetta *et al.*, 2015). The high cost of these studies and the increasing difficulties in obtaining permits for experimentation prompted researchers to look for other strategies. Many researchers have attempted to solve the problem by using *in vitro* cell systems (Alboni *et al.*, 2013b, 2014) that have many important advantages. Unfortunately,

the obtained results were often limited and inconclusive in elucidating the basis of diseases and identifying effective therapeutic strategies (Alberts, 2010). Invertebrates, thanks to their relatively simple nervous systems and to the latest technique in genome sequencing and manipulation, are becoming a useful tool for the study of neuronal physiology and for best disease process characterization (Ottaviani *et al.*, 2013; Tascetta *et al.*, 2015).

Invertebrates lack self-awareness “autonoetic consciousness” (Curren and Chalsani, 2012), emotional behaviour reduced to its individual components. In particular, the pond snail *Lymnaea stagnalis*, an aquatic pulmonate gastropod with a central nervous system (CNS) consisting of ≈20,000 neurons organized in a ring of interconnected ganglia, has proven to be an extremely useful and accessible model to study fundamental aspects of CNS function such as synaptic plasticity and associative memory. The serotonin (5-HT) neurons present in the CNS of *Lymnaea* are analogous to vertebrate 5-HT neurons that originate in the raphe nuclei. Serotonin, with specific innervation, control central pattern generators and other important circuits of the CNS. Furthermore, through integrated

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feedback information coming from the innervated areas, support general behavioural arousal (Andrianov *et al.*, 2015; Gillette, 2006).

In this model recent finding demonstrate a fundamental role of serotonin in the control of reproduction and behaviour (Il-Han *et al.* 2010, Ivashkin *et al.* 2015)

Dysfunction in serotonin system regulation or serotonin levels are involved in the control of many biological functions (Gillette 2006, Deneris *et al.* 2012). Understanding the transcription mechanisms associated with the hyper stimulation of the serotonergic system is a key step to clarifying the fundamental aspects of these mechanisms. In this context, we sought to mimic the generalized activation of the serotonergic system by administering the rate-limiting 5-HT precursor 5-hydroxytryptophan (5-HTP) to freely moving animals. We used 5-HTP because studies in rodents and molluscs have proven that exposure to 5-HT or tryptophan are less effective in elevating serotonin and related molecules, with shorter amplitude and duration. It is also possible that 5-HTP disrupt homeostatically regulated serotonin levels (Lynn-Bullock *et al.*, 2004; Marinesco *et al.*, 2004; Fickbohm *et al.*, 2005).

In this study, we evaluated the effects of 5-HTP on the serotonin related genes and on major intracellular systems related to serotonergic stimulation. The molecular machinery governing serotonin signaling has been cloned and characterized in *Lymnaea*. Serotonin is synthesized by tryptophan hydroxylase (LymTPH) (Koert *et al.*, 2001) in the cytoplasm of the presynaptic serotonergic neurons, vesicle monoamine transporters (LymVMAT) then package serotonin into vesicles, and upon fusion with the cell membrane, the neurotransmitter is released into the synaptic cleft and binds to specific receptors (LymHTR1 and LymHTR2) (Sugamori *et al.*, 1993; Gerhardt *et al.*, 1996). The concentration of 5HT is then regulated by the action of a specific transporter (LymSERT) (Sadamoto *et al.*, 2008).

The activity of serotonin receptors produces important intracellular changes related to cAMP and Ca⁺⁺ signalling (Poser *et al.* 2001). These pathways, in invertebrates and in mammals, are both linked to serotonergic control of related stress responses and adaptive mechanisms in response to pharmacological treatment (Kaang *et al.* 1993, Vinet *et al.*, 2003, 2004; Blom *et al.*, 2006).

One target for the serotonergic stimulation is a modifying effect on the regulation of postreceptor pathways and genes related to the cAMP cascade in particular the transcription factor cAMP response element binding protein (CREB) (Kaang *et al.*, 1993; Marinesco *et al.*, 2004). CREB is known to regulate the downstream expression of cAMP-inducible genes, and is proposed to be involved in the control of many biological functions, in the regulation of brain homeostasis and in the response to pharmacological treatment (Blom *et al.*, 2002; Alboni *et al.*, 2010, 2011, 2013a). In *Lymnaea*, analogue of CREB (LymCREB1) has been cloned and characterized (Sadamoto *et al.*, 2004).

Materials and Methods

Animals and colony maintenance

Laboratory-reared freshwater pond snails, *Lymnaea stagnalis* (original stocks donated by Vrije Universiteit, Amsterdam) were maintained in aquaria at the University of Modena and Reggio Emilia (Italy) in standard laboratory conditions: 21 - 23 °C, 12:12 h light/dark cycle (on at 08:00). Adult animals having shell lengths of 20 to 25 mm were used in this experiment and were kept in 12 L tanks (30 mature snails in each) supplied with well-aerated water. They were fed pesticide-free lettuce and goldfish pellets three times a week, and the aquaria were cleaned on alternate days. Every effort was made to minimize the number of animals used and their suffering.

Pharmacological experiments

The following compounds were used for pharmacological treatments: 5-hydroxy-L-tryptophan (5-HTP) 1 mM (Sigma-Aldrich); fluoxetine hydrochloride (FLX) 1 µM (Polichimica). Solutions of specified concentrations were freshly prepared in boiled filtered water (FW) with 50 µM ascorbic acid (Sigma-Aldrich) in order to avoid 5-HTP oxidation. We incubated adult snails for 6 or 48 h without food and aeration in 2 L aquaria, 15 specimens per 400 ml of experimental solution. Untreated controls (naive adults) were left undisturbed in an equal amount of FW, a group exposed only to ascorbic acid in the same conditions was also included. After incubation the animals were anesthetized on ice for 10 min and the central ring ganglia was dissected out (buccal ganglia were excluded) and stored at -80 °C prior analysis.

Total RNA extraction, reverse transcription, and real time polymerase chain reaction

Four central ring ganglia were pooled for total RNA extraction, 4 - 6 replicates were analyzed for each group. Total RNA extraction and DNase treatment were performed using GenElute™ Total RNA Miniprep Kit and DNASE70-On-Column DNase I Digestion Set (Sigma Aldrich) as previously described (Benatti *et al.*, 2011). Five hundred ng of total RNA was reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation) in 20 µl of reaction mix. mRNAs were quantified by real-time quantitative polymerase chain reaction in Roche LightCycler® 480 (Roche Diagnostics GmbH) using Power UP SYBR Green mix (Life Technologies Corporation). Specific forward and reverse primers were used at the final concentration of 300 nM (Table 1). Single PCR products were subjected to a heat dissociation protocol as previously described (Caraci *et al.*, 2016). Cycle threshold (Ct) value was determined by the LightCycler® 480 Software (Roche Diagnostics GmbH).

Statistical analysis

For quantitative evaluation of changes the comparative $\Delta\Delta C_t$ method was performed, using as calibrator the average levels of expression of control snails. The stability of mRNA expression of two reference genes (elongation factor 1-alpha, *LymEF1α*

Table 1 Nucleotide sequence of the forward and reverse primers used for Real-Time PCR

Gene Bank Accession	Target	Product Length	Type sequence	Ct (25 ng)
AB041522.1	<i>Lymnaea stagnalis</i> cAMP responsive element binding protein, <i>Lym</i> CREB1	180 bp [49-229]	Fw GTCAGCAGGGAATGGTCCTG Rv AACCGCAGCAACCCTAACAA	25
JX524180.1	<i>Lymnaea stagnalis</i> P2X receptor, <i>Lym</i> P2X	150bp [1005-1155]	Fw GGGATCGTCTTCGTGGTGA Rv AGTTCCTGGCCTTCAACAGAT	24
AJ238276.1	<i>Lymnaea stagnalis</i> neuropeptide Y, <i>Lym</i> NPY	188 bp [432-620]	Fw ACTCTTGGTGTCACTGCTCG Rv CTTGCGCCGTTTCTCTTTCC	17
L06803.1	<i>Lymnaea stagnalis</i> serotonin receptor 1, <i>Lym</i> HTR1	126 bp [893-1019]	Fw ACTATCTCATCCTGTCTTGG Rv GATATCCACATGTCACACAC	23
U50080.1	<i>Lymnaea stagnalis</i> serotonin receptor 2, <i>Lym</i> HTR2	115 bp [884-999]	Fw ACACCTGGAGTATTCTCATC Rv GAAGTAGTTGGTCACGTTCT	23
FX185022	<i>Lymnaea stagnalis</i> serotonin transporter, <i>Lym</i> SERT	177 bp [726-903]	Fw ATACCGTACCTTGTCATGTT RvTGTGTAGTACCAGGAGACA	20
AF129815.1	<i>Lymnaea stagnalis</i> tryptophan hydroxylase, <i>Lym</i> TPH	179 bp [238-417]	Fw AGGATACAGTCTACCGACAG Rv TGAGTTCACGGAAAATATT	18
AF484094.1	<i>Lymnaea stagnalis</i> vesicular monoamine transporter, <i>Lym</i> VMAT	172bp [529-701]	Fw AACGTGTACATGACTGTGAC Rv AAGCCAGTAAACATTGGTAT	22
DQ278441.1	<i>Lymnaea stagnalis</i> elongation factor 1-alpha, <i>Lym</i> EF1 α	150bp [7-157]	Fw GTGTAAGCAGCCCTCGAACT Rv TTCGCTCATCAATACCACCA	16
X15542.1	Snail, beta-tubulin, <i>Lym</i> TUB	127 bp [92-219]	Fw GAAATAGCACCGCCATCC Rv CGCCTCTGTGAAGTCCATCT	16

The accession number, the size (bp) of the PCR product obtained by amplification of the cDNA (mRNA) are given for each target. As indication of the relative abundances of each target average Ct values in adult snails (25 ng, n = 4).

and beta-tubulin, *Lym*TUB) was assessed using Normfinder®, *Lym*TUB was the most stable gene across groups and was used for gene normalization. Statistical analyses were performed using an analysis of variance (One-way ANOVA). Significant changes were determined by Tukey post-hoc test (with $p < 0.05$ significance level).

Results

Effect of an exposure to 5-HTP for 6 or 48 h on the expression levels of analogues of CREB1 and P2X in the CNS of L. stagnalis

Lymnaea CREB1 (*Lym*CREB1) is a homolog of mammalian CREB that is expressed in the CNS of *Lymnaea* and is involved in synaptic facilitation (Sadamoto *et al.*, 2004, 2010). One way ANOVA revealed a main effect of both an acute and a prolonged exposure to 5-HTP [F (4;21) = 5.282, $p = 0.004$ and F (4;30) = 13.896, $p < 0.0001$ respectively; Fig. 1A]. In particular, post hoc analysis showed that the expression of CREB1 was significantly induced in snails exposed for 48 h to 5-HTP with respect to all the other treatment regimens ($p < 0.001$). The effect of 5-HTP was reduced in presence of FLX: *Lym*CREB1 mRNA levels of the 5-HTP/FLX group were significantly higher than the control group ($p < 0.05$), while being significantly lower than the group receiving 5-HTP alone ($p < 0.01$). In contrast, following a 6 h exposure we observed a significant decrease of *Lym*CREB1

expression in the group exposed either to 5-HTP or FLX and to the combination of the two compounds with respect to control ($p < 0.05$) (Fig. 1A).

A similar trend was observed for *Lym*P2X (Fig. 1B); this purinergic receptor was recently identified and cloned in the CNS of *Lymnaea* (Bavan *et al.*, 2012). Prolonged exposure to 5-HTP resulted in an overall significant increase in *Lym*P2X mRNA in ganglia [F (4;30) = 22.506, $p < 0.0001$]. Again, this effect was still present when snails were exposed to both FLX and 5-HTP, but was significantly reduced with respect to the increase observed following 5-HTP alone ($p < 0.0001$) (Fig. 1B). When considering the effects of a 6 h treatment, one way ANOVA revealed a main effect [F (4;20) = 6.257, $p = 0.002$; Fig. 1B], indeed, P2X expression was reduced by 5-HTP, FLX, and their combination with respect to control ($p < 0.05$).

Effect of a 5-HTP exposure on the expression levels of components of the serotonergic system in the CNS of L. stagnalis

To date, two 5-HT receptor genes have been cloned in *Lymnaea*: *Lym*HTR1 and *Lym*HTR2 (Sugamori *et al.*, 1993; Gerhardt *et al.*, 1996). *Lym*HTR1 expression levels were affected by a 6 h exposure to 5-HTP [F (4;18) = 5.714, $p = 0.004$; Fig. 2A], while no effect was observed when treatment was protracted up to 48 h [F (4;29) = 1.609, $p = 0.199$; Fig. 3A]. In particular post hoc test revealed that FLX 1 μ M was able to decrease the expression

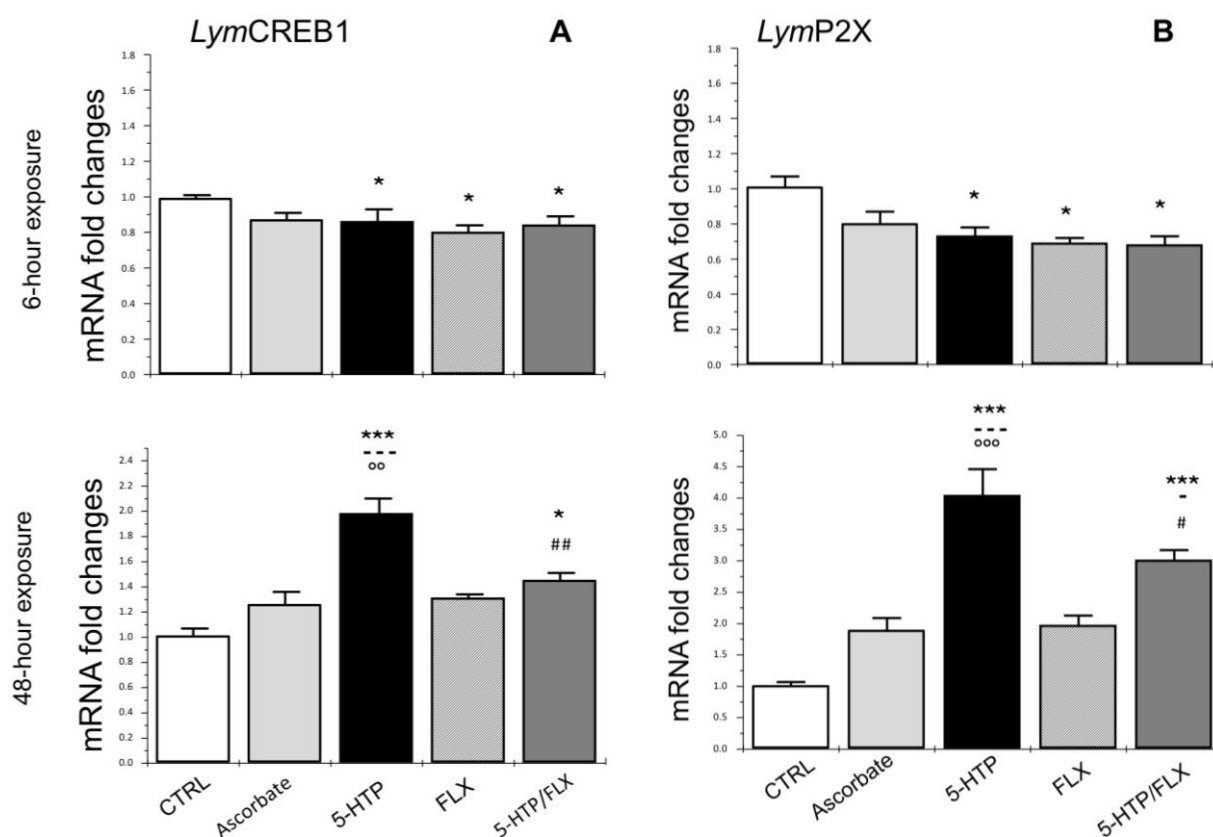


Fig. 1 Effect of an exposure to 5-HTP for 6 or 48 hs on the expression levels of analogues of CREB1 and P2X in the CNS of *Lymnaea stagnalis*. Adult snails were incubated in 1 mM 5-HTP, 1 μ M FLX, or a combination of the two (5-HTP/FLX) for 6 or 48 h. Untreated adults (CTRL) and a group exposed only to ascorbic acid (Ascorbate) in the same conditions were also included. *LymCREB1* (A) and *LymP2X* (B) mRNA expression in the ganglia, with *LymTUB* as endogenous control, were measured by Real-time PCR. N = 4 - 7 pools of 3 snails each. Data are represented as means \pm S.E.M. and were analyzed with ANOVA followed by Tukey. *** p < 0.0001, ** p < 0.01, * p < 0.05 vs CTRL; p < 0.0001, p < 0.05 vs Ascorbate; °°° p < 0.0001, °° p < 0.01 vs FLX; ## p < 0.01, # p < 0.05 vs 5-HTP.

levels of *LymHTR1* with respect to both control groups (p < 0.05). This down-regulation was blunted in the group exposed to 5-HTP, alone (p = 0.072) or in combination with FLX (p = 0.015). On the other hand, the expression levels of the other serotonergic receptor, *LymHTR2*, were not altered on our experimental conditions [F (4;19) = 1.140, p = 0.368 for 6 h; Fig. 2B, and F (4;29) = 2.380, p = 0.075 for 48 h exposure; Fig. 3B].

Sadamoto and co-workers (2008) identified and characterized the localization of the of the serotonin transporter in *Lymnaea*: *LymSERT*. In our experimental conditions, no effect on *LymSERT* mRNA levels was observed following a 6 h treatment regime [F (4;21) = 2.010, p = 0.130; Fig. 2C], while exposure to 5-HTP for 48 h significantly increased the expression levels of serotonin transporter with respect to untreated controls [F (4;29) = 3.043, p = 0.033; Fig. 3C].

We also evaluated in ganglia the transcriptional effect of a serotonergic stimulation on the rate-limiting enzyme in the synthesis of serotonin: tryptophan hydroxylase (*LymTPH*) (Koert *et al.*,

2001). A main effect was revealed in animals experiencing a 48 h exposure to 5-HTP [F (4;30) = 8.662, p < 0.0001; Fig. 3D], while no effect was observed following a 6 h treatment [F (4;18) = 1.887, p = 0.157; Fig. 2D]. In particular, *LymTPH* mRNA was significantly higher in the ganglia of ascorbic acid-exposed snail with respect to the levels found in both the 5-HTP exposed groups (alone (p = 0.007) or in combination with FLX (p < 0.0001) and in untreated control snails (p = 0.017) (Fig. 3D).

Vesicular monoamine transporter (*LymVMAT*) mRNA levels were not affected in snails exposed to 5-HTP, FLX or their combination for 6 h with respect to control groups [F (4;19) = 2.058, p = 0.127] (Fig. 2E). On the other hand, one-way ANOVA revealed a main effect of a chronic treatment with 5-hydroxytryptophan on VMAT expression [F (4;26) = 11.300, p < 0.0001] (Fig. 3E). We observed a significant decrease of *LymVMAT* mRNA in animals exposed to 5-HTP for 48 h, alone or in combination to FLX, with respect to control groups (p < 0.01) or to FLX-treated snails (p < 0.01).

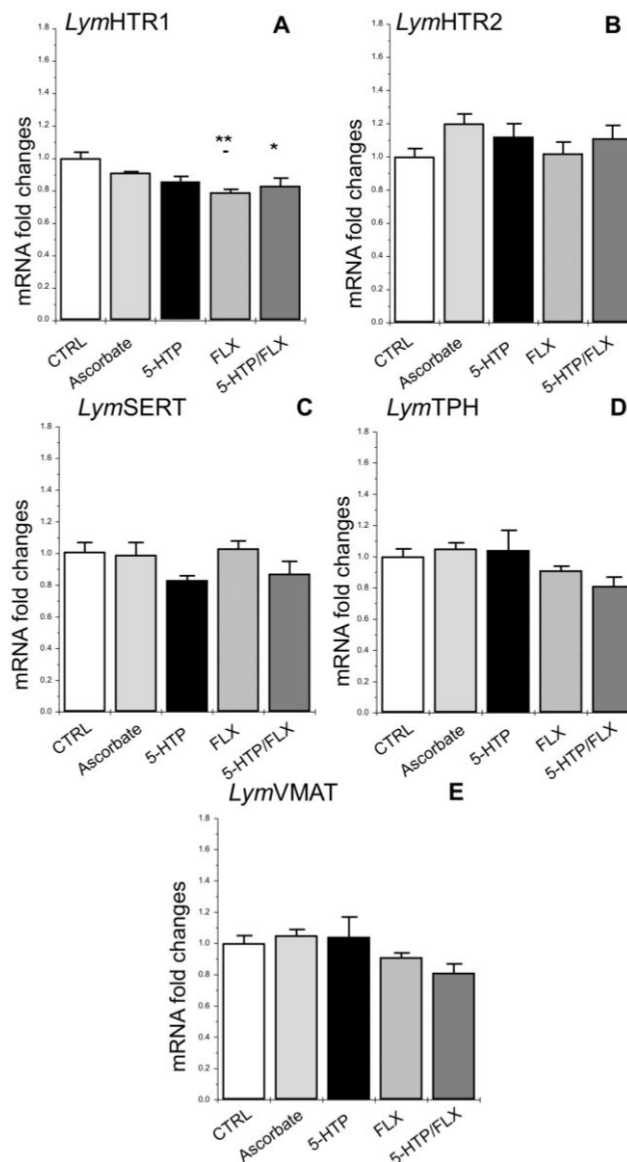


Fig. 2 Effect of a 6-h 5-HTP exposure on the expression levels of components of the serotonergic system in the CNS of *Lymnaea stagnalis*. Adult snails were incubated in 1 mM 5-HTP, 1 μ M FLX or a combination of the two (5-HTP/FLX) for 6 h. Untreated adults (CTRL) and a group exposed only to ascorbic acid (Ascorbate) in the same conditions were also included. *Lymnaea stagnalis* serotonin receptors [*LymHTR1* (A), *LymHTR2* (B)] and transporter [*LymSERT* (C)], tryptophan hydroxylase [*LymTPH* (D)], vesicular monoamine transporter [*LymVMAT* (E)] mRNA expression in the ganglia, with *LymTUB* as endogenous control, were measured by Real-time PCR. N = 4 - 7 pools of 3 snails each. Data are represented as means \pm S.E.M. and were analyzed with ANOVA followed by Tukey. ** $p < 0.01$, * $p < 0.05$ vs CTRL; $p < 0.05$ vs Ascorbate.

Discussion

Here we demonstrated that specific transcription was strongly induced following a prolonged, but not an acute, exposure to 5-HTP in the ganglia of *Lymnaea*. In particular, *LymCREB1* and *LymP2X* mRNA levels were increased in snails receiving 5-HTP for 48 h, and decreased following a 6 h exposure. Interestingly, this effect was reduced when snails were exposed chronically to both 5-HTP and FLX, suggesting a role for SERT in regulating the effects of 5-HTP.

Previous studies in vertebrates and invertebrates have shown that a treatment with 5-HTP is able to increase serotonin content in the CNS, in both serotonergic and non-serotonergic regions (Gartside *et al.*, 1992; Lynn-Bullock *et al.*, 2004; Fickbohm *et al.*, 2005). Moreover, studies on isolated neuron in *Lymnaea* have demonstrated that the precursor acts only indirectly through its conversion to serotonin and its effects are mediated by enhanced serotonin release, activation of its receptors and modulation of electrical activity (Dyakonova *et al.*, 2009).

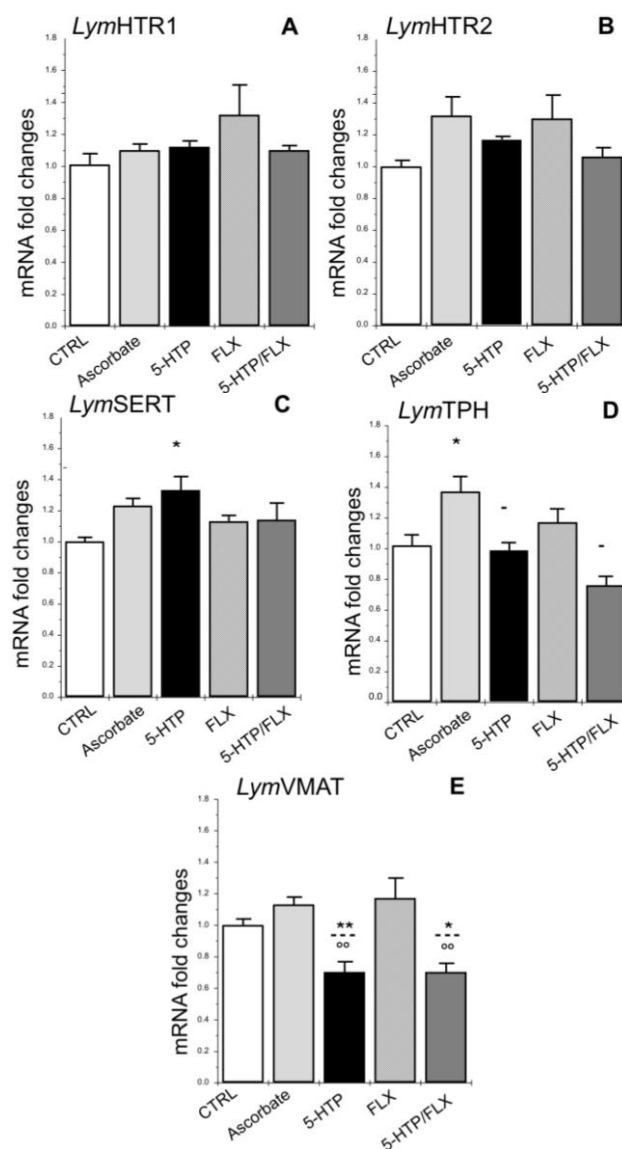


Fig. 3 Effect of a 48-h 5-HTP exposure on the expression levels of components of the serotonergic system in the CNS of *Lymnaea stagnalis*. Adult snails were incubated in 1 mM 5-HTP, 1 μ M FLX or a combination of the two (5-HTP/FLX) for 48 h. Untreated adults (CTRL) and a group exposed only to ascorbic acid (ASC) in the same conditions were also included. *Lymnaea stagnalis* serotonin receptors [*LymHTR1* (A), *LymHTR2* (B)] and transporter [*LymSERT* (C)], tryptophan hydroxylase [*LymTPH* (D)], vesicular monoamine transporter [*LymVMAT* (E)] mRNA expression in the ganglia, with *LymTUB* as endogenous control, were measured by Real-time PCR. N = 4 - 7 pools of 3 snails each. Data are represented as means \pm S.E.M. and were analyzed with ANOVA followed by Tukey. ** $p < 0.01$, * $p < 0.05$ vs CTRL; $p < 0.0001$, $p < 0.05$ vs Ascorbate; °° $p < 0.01$ vs FLX.

In our experimental conditions we demonstrated that 48 h exposure to serotonin precursor is able to influence also gene expression of selected targets and that some of these effects were significantly diminished in the presence of FLX, a selective inhibitor of serotonin transporter. In the group exposed to FLX alone for 48 h no alterations of gene expression of the evaluated targets were observed. This effect is in agreement with the results of Yu RL and collaborators (2008) that have shown that in *Aplysia*, 24 h treatment of

paired pleural-pedal ganglia, induced the mRNA of CREB gene and protein.

In *Aplysia*, treatment with 5-HTP has been demonstrated to potentiate serotonergic activity (Marinesco *et al.*, 2004), which, in turn, may increase intracellular levels of cAMP and cause a rapid and transient induction of CRE-responsive genes (Kaang *et al.*, 1993). This effect is mediated by the phosphorylation of CREB at Ser¹¹⁹, this transcription factor is a key component in regulating synaptic plasticity both in physiologic and pathologic

conditions (Kaang *et al.*, 1993; Sadamoto *et al.* 2010, Blom *et al.*, 2002; Alboni *et al.*, 2011). We observe a strong increase in CREB mRNA levels following a 48 h serotonergic stimulation in the CNS of *Lymnaea* which could be the basis for major behavioral changes induced by serotonin in different invertebrate models (Il-Han *et al.*, 2011; Andrianov *et al.*, 2015).

The LymP2x gene acts similarly to CREB. Currently, in the literature, there is no direct evidence of a link between the transcription of this receptor and the activity of the serotonergic system. We can hypothesize that an increase in the number of receptors can lead to an increase in intracellular Ca⁺⁺ and a strengthening of CREB's transcriptional activity. The link between CREB and Ca⁺⁺ is widely demonstrated both in mammalian and invertebrate models (Poser and Storm 2001; Ghosh-Roy *et al.*, 2010).

Studies on knockout mice have established the importance of VMAT2 in regulating catecholamine and serotonin levels, and their release from neurons following a depolarizing stimulus (Eiden and Weihe, 2011). Similarly, in our model a long lasting serotonergic stimulation caused a significant down-regulation in LymVMAT expression, this effect was still present even when snails were exposed to the combination 5-HTP/FLX, suggesting that this effect does not depend on SERT functionality. It is possible that the increased activity of serotonergic neurons evoked by exposure to 5-HTP may induce this down-regulation.

Conclusions

Our experiments show that stimulation of the serotonergic system can induce specific transcriptional changes in the ganglia of *L. stagnalis*. Furthermore, these data suggest that *Lymnaea* ideally suited to unravel the complexity of the serotonin signaling pathway and may represent a good model to provide new insights on how serotonin can modulate different biological functions and its role in brain homeostasis.

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References

Alberts B. Model organisms and human health. *Science* 330: 1724, 2010.

Alboni S, Benatti C, Capone G, Corsini D, Caggia F, Tascedda F, *et al.* Time-dependent effects of escitalopram on brain derived neurotrophic factor (BDNF) and neuroplasticity related targets in the central nervous system of rats. *Eur. J. Pharmacol.* 643: 180-187, 2010.

Alboni S, Tascedda F, Corsini D, Benatti C, Caggia F, Capone G, *et al.* Stress induces altered CRE/CREB pathway activity and BDNF expression in the hippocampus of glucocorticoid receptor-impaired mice. *Neuropharmacology* 60: 1337-1346, 2011.

Alboni S, Benatti C, Montanari C, Tascedda F, Brunello N, Chronic antidepressant treatments resulted in altered expression of genes involved in inflammation in the rat hypothalamus. *Eur. J. Pharmacol.* 721: 158-167, 2013a.

Alboni S, Gibellini L, Montanari C, Benatti C, Benatti S, Tascedda F, *et al.* N-acetyl-cysteine prevents toxic oxidative effects induced by IFN- α in human neurons. *Int. J. Neuropsychopharmacol.* 16: 1849-1865, 2013b.

Alboni S, Montanari C, Benatti C, Sanchez-Alavez M, Rigillo G, Blom JM, *et al.* Interleukin 18 activates MAPKs and STAT3 but not NF- κ B in hippocampal HT-22 cells. *Brain Behav. Immun.* 40: 85-94, 2014.

Andrianov VV, Bogodvid TK, Deryabina IB, Golovchenko AN, Muranova LN, Tagirova RR, *et al.* Modulation of defensive reflex conditioning in snails by serotonin. *Front. Behav. Neurosci.* 9: 279, 2015.

Bavan S, Straub VA, Webb TE, Ennion SJ,. Cloning and characterization of a P2X receptor expressed in the central nervous system of *Lymnaea stagnalis*. *PLOS ONE* 7: e50487, 2012.

Benatti C, Alboni S, Montanari C, Caggia F, Tascedda F, Brunello N, *et al.* Central effects of a local inflammation in three commonly used mouse strains with a different anxious phenotype. *Behav. Brain Res.* 224: 23-34, 2011.

Blom JMC, Tascedda F, Carra S, Ferraguti C, Barden N, Brunello N. Altered regulation of CREB by chronic antidepressant administration in the brain of transgenic mice with impaired glucocorticoid receptor function. *Neuropsychopharmacology* 26: 605-614, 2002.

Blom JM, Benatti C, Alboni S, Capone G, Ferraguti C, Brunello N, *et al.* Early postnatal chronic inflammation produces long-term changes in pain behavior and N-methyl-D-aspartate receptor subtype gene expression in the central nervous system of adult mice. *J. Neurosci. Res.* 84: 1789-1798, 2006.

Caraci F, Tascedda F, Merlo S, Benatti C, Spampinato SF, Munafò A, *et al.* Fluoxetine Prevents A β 1-42-Induced Toxicity via a Paracrine Signaling Mediated by Transforming-Growth-Factor- β 1. *Front. Pharmacol.* 7: 389, 2016.

Curran KP, and Chalasani SH, Serotonin Circuits and Anxiety: What Can Invertebrates Teach Us. *Invert. Neurosci.* 12: 81-92, 2012.

Dyakonova VE, Chistopolsky IA, Dyakonova TL, Vorontsov DD, Sakharov DA. Direct and decarboxylation-dependent effects of neurotransmitter precursors on firing of isolated monoaminergic neurons. *J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol.* 195: 515-527, 2009.

Eiden LE, Weihe E, VMAT2: a dynamic regulator of brain monoaminergic neuronal function interacting with drugs of abuse. *Ann. NY Acad. Sci.* 1216: 86-98, 2011.

Fickbohm DJ, Spitzer N, Katz PS. Pharmacological manipulation of serotonin levels in the nervous system of the opisthobranch mollusc *Tritonia diomedea*. *Biol. Bull.* 209: 67-74, 2005.

- Gartside SE, Cowen PJ, Sharp T. Effect of 5-hydroxy-L-tryptophan on the release of 5-HT in rat hypothalamus in vivo as measured by microdialysis. *Neuropharmacology* 31: 9-14, 1992.
- Gerhardt CC, Leysen JE, Planta RJ, Vreugdenhil E, Van Heerikhuizen H. Functional characterisation of a 5-HT₂ receptor cDNA cloned from *Lymnaea stagnalis*. *Eur. J. Pharmacol.* 311: 249-258, 1996.
- Ghosh-Roy A, Wu Z, Goncharov A, Jin A, Chisholm AD. Calcium and cyclic AMP promote axonal regeneration in *Caenorhabditis elegans* and Require DLK-1 Kinase. *J. Neurosci.* 30: 3175-3183, 2010.
- Gillette R. Evolution and function in serotonergic systems. *Integr. Comp. Biol.* 46: 838-846, 2006.
- Il-Han J, Janes T, Lukowiak K. The role of serotonin in the enhancement of long-term memory resulting from predator detection in *Lymnaea*. *J. Exp. Biol.* 213: 3603-3614, 2010.
- Kaang BK, Kandel ER, Grant SGN. Activation of cAMP-Responsive genes by stimuli that produce long-term facilitation in aplysia sensory neurons. *Neuron* 10: 427-435, 1993.
- Koert CE, Spencer GE, van Minnen J, Li KW, Geraerts WP, Syed NI, *et al.* Functional implications of neurotransmitter expression during axonal regeneration: serotonin, but not peptides, auto-regulate axon growth of an identified central neuron. *J. Neurosci.* 21: 5597-5606, 2001.
- Ivashkin E, Khabarova MY, Melnikova V, Nezlin, LP, Kharchenko O, Voronezhskaya EE, *et al.* Serotonin mediates maternal effects and directs developmental and behavioral changes in the progeny of snails. *Cell Reports* 12: 1144-1158, 2015.
- Lynn-Bullock CP, Welshhans K, Pallas SL, Katz PS. The effect of oral 5-HTP administration on 5-HTP and 5-HT immunoreactivity in monoaminergic brain regions of rats. *J. Chem. Neuroanat.* 27: 129-138, 2004.
- Marinesco S, Wickremasinghe N, Kolkman KE, Carew TJ. Serotonergic modulation in *aplysia*. II. Cellular and behavioral consequences of increased serotonergic tone. *J. Neurophysiol.* 92: 2487-2496, 2004.
- Ottaviani E, Accorsi A, Rigillo G, Malagoli D, Blom JMC, Tascedda F. Epigenetic modification in neurons of the mollusc *Pomacea canaliculata* after immune challenge. *Brain Res.* 1537: 18-26, 2013.
- Poser S, Storm DR. Role of Ca²⁺-stimulated adenylyl cyclases in LTP and memory formation. *Int. J. Dev. Neurosci.* 19: 387-394, 2001.
- Sadamoto H, Kitahashi T, Fujito Y, Ito E. Learning-dependent gene expression of CREB1 isoforms in the molluscan brain. *Front. Behav. Neurosci.* 4: 25, 2010.
- Sadamoto H, Sato H, Kobayashi S, Murakami J, Aonuma H, Ando H, *et al.* CREB in the pond snail *Lymnaea stagnalis*: cloning, gene expression, and function in identifiable neurons of the central nervous system. *J. Neurobiol.* 58: 455-466, 2004.
- Sadamoto H, Serfozo Z, Ito E. Localization of serotonin transporter mRNA in the CNS of *Lymnaea stagnalis*. *Acta Biol. Hung.* 59: 61-64, 2008.
- Sugamori KS, Sunahara RK, Guan HC, Bulloch AG, Tensen CP, Seeman P, *et al.* Serotonin receptor cDNA cloned from *Lymnaea stagnalis*. *Proc. Natl. Acad. Sci. USA* 90: 11-15, 1993.
- Tascedda F, Malagoli D, Accorsi A, Rigillo G, Blom JMC, Ottaviani E. Molluscs as models for translational medicine. *Med. Sci. Monit. Basic Res.* 21: 96-99, 2015.
- Vinet J, Carra S, Blom JMC, Harvey M, Brunello N, Barden N, *et al.* Cloning of mouse Ca²⁺/calmodulin-dependent protein kinase kinase beta (CaMKK β) and characterization of CaMKK β and CaMKK α distribution in the adult mouse brain. *Brain Res. Mol. Brain Res.* 111: 216-221, 2003.
- Vinet J, Carra S, Blom JMC, Harvey M, Brunello N, Barden N, *et al.* Chronic treatment with desipramine and fluoxetine modulate BDNF, CaMKK α and CaMKK β mRNA levels in the hippocampus of transgenic mice expressing antisense RNA against the glucocorticoid receptor. *Neuropharmacology* 47: 1062-1069, 2004.
- Yu RL, Fioravante D, Shah S, Byrne JH. cAMP response element-binding protein 1 feedback loop is necessary for consolidation of long-term synaptic facilitation in *Aplysia*. *J. Neurosci.* 20: 1970-1976, 2000.